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SPONTANEOUS MUTATION AT THE *R* LOCUS IN MAIZE

III. GENETIC MODIFICATION OF MUTATION RATE

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AMONG R^r stocks tested for frequency of seed-color mutation, the "Cornell stock" showed the highest mutation rate, 18.2×10^{-4} (Stadler, 1948). Extracted stocks homozygous for R^r: Cornell, derived from crosses of the Cornell stock with other stocks, showed that this allele is not unusually mutable, as compared with other R^r alleles. Its high mutation rate in the Cornell stock is therefore due to the presence in this stock of modifying genes favorable to its mutation. The present paper is concerned with an attempt to identify genes modifying the mutation rate of R^r: Cornell.

A multiple linkage tester stock, *B a su pr Y-Pl J wx* R^g, was crossed with the Cornell stock (*b A Su Pr y-pl j Wx* R^r). The marker genes listed are located respectively on chromosomes 2, 3, 4, 5, 6, 8, 9, and 10. The F_1 progeny, tested for seed-color mutation rate of *R*, yielded no mutations among the 24,339 female gametes tested. Since the F_1 plants tested for mutation were R^r R^g in constitution, about half

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of the gametes tested were R^r: Cornell gametes. In a population of 12,170 gametes, in the Cornell stock, the expected yield of seed-color mutations would be 22. Thus the total effect of the genes of the tester stock which differed from those of the Cornell stock, as shown in plants which were heterozygous for all these genes, was a pronounced reduction in the frequency of mutation of the R^r: Cornell allele.

The part played in this effect by each of the chromosome-segments bearing a marker gene (except for the segment marked by *a*) may be estimated in the progeny produced by backcrossing the F_1 to the Cornell stock. For example, considering only the segment marked by *B*, half of the plants of the backcross progeny will be *B b* and half *b b*. Now if a factor *M*, reducing the mutation frequency of R^r, is located in the segment marked by *B*, it will tend to be present in the backcross progeny in plants of the *B b* group, and to be absent in plants of the *b b* group. It will thus tend to result in a lower frequency of mutation in the former group than in the latter. Plants in which *M* is present will in all cases be heterozygous for this factor, for all plants of the backcross progeny will carry also the contrasting allele from the Cornell stock. Thus the backcross can identify only factors derived from the tester stock which are dominant to the corresponding factors present in the Cornell stock. Dominant factors increasing mutation frequency, derived from the tester stock, would be identified similarly, by increased rather than decreased mutation-frequency in the group identified by the marker gene (the *B b* group in the example given). The term "dominant" is here used in the broadest sense, to apply to any allelic relation in which the heterozygote *B b* differs recognizably from the homozygote *b b*. It is not implied that the effect of *B b* is more like that of *B B* than that of *b b*, for in this experiment it is not feasible to determine the homozygous effect of the mutation modifier derived from the tester stock.

The same backcross progeny may be classified similarly

for the other segregating marker-genes, for evidence of mutation modifiers associated with the chromosome segments marked. For each of the dominant plant-character genes of the tester stock, *B*, *Pl* and *J*, the two classes of backcross individuals concerned are readily identified by inspection of the backcross plants. For the marker genes of which the tester stock carries a recessive allele (*i.e.*, *su*, *pr*, *y*, *wx*, R^g), the two classes of backcross individuals are the homozygous dominant and the heterozygote (*e.g.*, *Su Su* and *Su su*). Since these genes are identified by seed or seedling characters, the two classes may be made distinguishable by using as the pollen stock, in the detasseled plot in which mutation frequency is determined, a stock recessive or heterozygous at each of these loci. The pollen stock used was r^g *Su su pr y wx*. It was therefore possible, by inspection of the ears, to classify the backcross plants for *su*, *pr*, *Y*, and *wx*. Seedling cultures, grown from 20-seed samples from each plant, permitted the further classification of the backcross plants as to R^g ($R^r R^r$ vs. $R^r R^g$). Classification for *a* could not be accomplished in this way, since its phenotypic effect conflicts with that of *r*. The addition of *a a* or *A a* to the pollen stock used in the detasseled plot would have made it impossible to detect the seed-color mutations of *R*.

Each of the plants of the backcross progeny could thus be classified as to the presence or absence of a chromosome segment from the tester stock, in the case of each of seven genetically marked segments, one of which was marked by the two genes *Y* and *Pl*. The data on mutation frequency in the 224 plants of the backcross progeny could then be classified successively as to each of the eight marker-genes, to determine any differences in mutation rate associated with the presence of these chromosome segments.

Obviously, a factor modifying the mutation rate will be detectable in the backcross progeny only if it has a pronounced effect upon mutation frequency. The effect must be large enough to produce a significant difference in mutation rate in the contrasted classes, in the face of the high

sampling error inevitably associated with mutation rates. Consider, for example, a backcross progeny yielding 30 mutations, the number actually identified in the backcross progeny used in this study. Suppose that one-half of the gametes tested are from *B b* plants, and one-half from *b b* plants. How large a difference in mutation frequency is required for reasonable assurance that the difference observed is not due to sampling fluctuations? Assuming that a mutation-modifier closely linked with *B* actually doubles the mutation rate, and that twice as many mutations are identified in *B b* plants as in *b b* plants, the difference observed would be statistically insignificant. The observed result in this population would be 20 mutations in one class versus 10 in the other. A deviation from equality as large as this would occur by chance, if mutation were equally probable in the two classes, with a probability slightly greater than 0.05, and the result would therefore be considered statistically insignificant by conventional standards.

The effect of crossing over between the mutation-modifying gene and the marker gene is to reduce the detectable effect, and thus to reduce the probability of detection. For example, suppose that a modifier *M* results on the average in a ten-fold increase in mutation rate, and suppose that the modifier is linked with *B* with 20 per cent. crossing over. The comparison in mutation rate is made between *B b* plants and *b b* plants. Assuming for illustration uniform progenies of 100 seeds per plant and a uniform mutation rate of 0.1 per cent., except for the action of this modifier, the result would be as follows:

	B b plants			*b b* plants		
	No.	Genotype	Mut. Frequency	No.	Genotype	Mut. Frequency
Non-Crossover	80	B M/b m	80/ 8,000	80	b m/b m	8/ 8,000
Crossover	20	B m/b m	2/ 2,000	20	b M/b m	20/ 2,000
Total			82/10,000			28/10,000

Thus the 10-fold increase in mutation rate, which would be evident if there were no crossing over between the modifier and the marker gene, is reduced to less than a 3-fold

increase, as shown by the comparison of *B b* and *b b* plants. The minimal increase required to make a modifying factor detectable in the experiment applies of course to its net effect after this reduction has been made.

Accordingly, it may be expected that the comparison of mutation rates in the contrasted classes of the backcross progeny will identify only a small proportion of the factors which may affect the mutation of the gene studied. Among the gene differences occurring in the particular cross studied, it can detect only those in which the allele present in the tester parent is dominant to that present in the Cornell stock, only those of relatively large effect upon mutation frequency, and only those rather closely linked to the marker genes used.

Experimental Results

The backcross progeny was tested for seed-color mutation rate in three cultures, 59–101, 102, and 103, each grown from a single ear of $F_1 \times$ Cornell stock. These cultures comprised a total of 224 plants, bearing 41,600 seeds. Among the $R^r R^r$ plants of these cultures, each seed tests an R^r: Cornell gamete; among the $R^r R^g$ plants approximately half of the seeds are tests of R^r: Cornell gametes and half are tests of R^g gametes. The number of colorless seeds found was 32. Each of these, except two that failed to germinate, was confirmed as a mutation by subsequent test. All 30 mutants identified proved to be mutations to r^r, and were therefore presumably the result of mutation in R^r: Cornell gametes.

The estimated number of R^r: Cornell gametes tested in the backcross progenies (number of seeds of $R^r R^r$ plants plus half the number of seeds of $R^r R^g$ plants) was 32,457. The mutation rate for R^r: Cornell gametes in the backcross plants was thus 9.2×10^{-4} (or at most, including the untested mutants, 9.9×10^{-4}), approximately one-half the rate found in the Cornell stock. The 30 mutants observed were produced by 22 plants, the observed mutation fre-

quency in plants that gave more than a single mutant being 3/130, 3/682, 2/76, 2/240, 2/413, and 2/468.

The distribution of the mutants with relation to the segregating marker-genes is shown in Table 1. The same population of 32,457 R^r:Cornell gametes is here classified for each of the 8 marker-genes, except in cases in which the genotype was indeterminate. In such cases the plants concerned are omitted. The only case of serious difficulty in classification was for *j* among plants with *B*. The expression of *j* (japonica striping) in these cultures was restricted almost wholly to the leaf sheaths, and the development of anthocyanin in the leaf sheaths in plants of the *B b* class made classification for *j* doubtful. The *j* classification was therefore made only in the *b b* class.

TABLE 1

MUTATION FREQUENCY OF *Rr*:CORNELL IN RELATION TO LINKAGE-TESTER GENES

FIRST BACKCROSS

b Su Pr y-pl j Wx Rr (Cornell stock) /*B su pr Y-Pl J wx Rg* × Cornell stock

Genotype	59–101		59–102		59–103		Total		Mutation rate	L. E.
	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency		
									(× 10⁻⁴)	
B b	30	4/4,270	37	4/5,504	32	4/3,887	99	12/13,661	8.8	15.3–4.5
b b	37	6/4,900	44	8/6,396	42	4/7,059	123	18/18,355	9.8	15.5–5.9
Su Su	42	9/6,279	40	4/6,159	40	4/6,295	122	17/18,733	9.1	14.5–5.3
Su su	25	1/2,891	42	8/5,890	35	4/4,943	102	13/13,724	9.5	16.2–5.0
Pr Pr	34	3/4,232	45	7/6,516	28	3/4,291	107	13/15,039	8.6	14.8–4.6
Pr pr	33	7/4,938	37	5/5,533	47	5/6,947	117	17/17,418	9.8	15.6–5.7
Y y	33	6/5,138	42	9/6,361	37	7/6,145	112	22/17,644	12.5	18.9–7.8
y y	34	4/4,032	40	3/5,688	38	1/5,093	112	8/14,813	5.4	10.6–2.3
Pl pl	27	7/4,424	42	8/6,845	40	4/5,447	109	19/16,716	11.4	17.8–6.8
pl pl	40	3/4,746	40	4/5,204	34	4/5,775	114	11/15,725	7.0	12.5–3.5
J j	17	6/2,490	25	7/4,485	25	3/4,329	67	16/11,304	14.2	23.0–8.1
j j	20	0/2,410	16	0/1,643	16	1/2,698	52	1/ 6,751	1.5	8.3–0.0
Wx Wx	32	5/4,362	39	7/5,742	32	6/4,807	103	18/14,911	12.1	19.0–7.2
Wx wx	35	5/4,808	43	5/6,307	43	2/6,431	121	12/17,546	6.8	12.0–3.5
R^r R^r	36	10/6,133	47	9/8,802	46	8/8,378	129	27/23,313	11.6	16.9–7.6
R^r R^g	31	0/3,037	35	3/3,247	29	0/2,860	95	3/ 9,144	3.3	9.6–0.7

The results show suggestive differences in mutation rate associated with the marker genes *J*, R^g and *Y*, and possible differences of smaller extent asociated with *Pl* and *wx*.

The statistical significance of these differences may be estimated by the use of Stevens' table of the limits of the

expectation, for the binomial and Poisson distributions (Stevens, 1945) as described in the preceding paper. The customary method of estimating sampling fluctuations by the use of the standard error may be very inaccurate in the case of mutation rates, as Stevens (1942) has shown, since the assumption of normality of the distributions is not warranted. This error may be avoided by using the actual ranges expected, as determined from the binomial distribution. The limits of the expectation for various values of P are given in the table cited.

The method of estimating the significance of differences in mutation rate in the contrasted classes may be illustrated as follows, in the case of the comparison of *Y y* vs. *y y* plants: If the difference in genotype were without effect upon mutation of R^r, the expected number of mutants in the *Y y* class would be 16.3 ($30 \times 17{,}644/32{,}457$); in the *y y* class 13.7. The numbers of mutants observed in the two classes were 22 and 8, respectively. We may determine directly from the table whether the smaller class (the *y y* class in this case) has significantly less than its proportionate share of the total of 30 mutants. According to the table, for $n = 8$ and for $p = 8/30 = .267$, the limits of the expectation at the $P = .05$ level of significance are 3.7–13.8. The expected number of mutations, 13.7, is thus barely within the limits of sampling fluctuation at this level of probability, and the difference therefore is not quite significant by conventional standards.

The differences associated with *Pl* and *wx* are much less significant, the former being within the limits of sampling fluctuation at the $P = .2$ level, and the latter only slightly beyond these limits.

The difference associated with *j* is highly significant, as judged by this standard. The observed mutation rate in *J j* plants was 14.2, and in *j j* plants was 1.5. The expected number of mutants in the *j j* class is beyond the limit of the expectation not only at the $P = .05$ level but at the $P = .01$ level. The indication of a nearly 10-fold increase in mutation rate associated with *J* is of course very approxi-

mate, since the mutation rate in *j j* plants is based upon a single mutation, and is thus subject to a very high sampling error.

The difference associated with R^g is also significant. The mutation frequency observed in $R^r R^g$ plants was less than one-third as high as that in $R^r R^r$ plants. The expected number of mutants in the $R^r R^g$ class is beyond the limit of expectation at the P = .05 level, but not at the P = .01 level.

There may still be some material underestimate of sampling error in the determinations of significance given above. These determinations would be strictly accurate for samples from a homogeneous population. Obviously a population in which genetic comparisons are made can not be homogeneous, and comparisons to determine the effect of a single factor or complex can be made only by including in each class a large enough number of plants to reduce the effect of other segregating factors to a negligible quantity. The backcross progeny with which we are here concerned is apparently segregating for large differences in mutation rate linked with *J*, R^g, and possibly *Y*, and presumably also for similar differences determined by other chromosome segments not genetically marked. If these effects are cumulative, there must be very large differences in potential mutation rate between the individual plants. The extent to which plant variability reduces the significance of the differences is not measured in the estimates given above. To illustrate this error, suppose that in the data for *Y y* vs. *y y* plants, the 17,644 gametes tested to represent the *Y y* group came from 4 plants of the backcross progeny, while the 14,813 gametes representing the *y y* group came from 4 others. This would be technically possible if male gametes rather than female gametes were under test. Now obviously a major factor determining the result of the comparison would be the chance selection of the four plants in each group. The significance of a difference between the two groups could not be estimated without considering the variations in mutation rate in the individual plants. When the

test is made on female gametes, and thus limited to a small and variable number of gametes per plant, the mutation rates of individual plants are determined from quite insignificant samples, and therefore a significant estimate of the sampling error arising from individual plant variability can not be made. It may be supposed that the sampling error from plant variability is negligible when the rates in the two classes are based, as in this case, on the results from more than 100 plants each. But this is only a guess, since the extent of the plant variability is unknown. It should be noted also that, in the case of the difference associated with *J*, the number of plants tested was only about half as large as in the other comparisons.

Since later trials confirmed the significance of the *Y* effect, the relation of mutation frequency to the various combinations of *Y* and *Pl* is of interest. This is shown in Table 2.

TABLE 2

MUTATION FREQUENCY OF *Pr*:CORNELL IN RELATION TO *Y* AND *Pl*

FIRST BACKCROSS

	59–101		59–102		59–103		Total			
	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	Mutation rate	L. E.
									(× 10⁻⁴)	
Y Pl/y pl	20	6/3,410	30	7/4,660	27	4/4,247	77	17/12,317	13.8	22.1–8.0
Y pl/y pl	13	0/1,728	12	2/1,701	10	3/1,898	17	5/ 5,327	9.4	22.0–3.0
y Pl/y pl	7	1/1,014	12	1/2,185	13	0/1,200	32	2/ 4,399	4.5	16.4–0.6
y pl/y pl	27	3/3,018	28	2/3,503	25	1/3,893	80	6/10,414	5.8	12.5–2.1

If it may be assumed that the effect is due to a single modifier gene, the data suggest that this gene is closer to *Y* than to *Pl*. The higher mutation rate associated with *Pl* is due entirely to the plants which received from the F_1 parent a *Y-Pl* chromosome; those which received a *y Pl* crossover chromosome show no increase in mutation rate over those which received the *y pl* chromosome.

There is some slight indication that the gene-sequence is *Y-M*$_y$*-Pl* (using the symbol *M*$_y$ to designate the postulated mutation modifier). The reduced mutation rate in plants

which receive the *Y pl* crossover chromosome, in contrast to those with *Y Pl*, is in agreement with this supposition. But since the reduction is quite insignificant, the gene-sequence M_y-*Y*-*Pl* is almost equally likely.

The fact that the 7 chromosome segments tested included three with pronounced effects upon mutation frequency must mean that there is a rather large number of genes with such effects, for these 7 segments represent a rather small sample of the total genotype. The fact that the number of modifiers is large does not mean that the effect of the individual modifier must be small, since the effects may occur in either direction. Actually the results indicate that the effects of the single modifier may be surprisingly large.

It is noteworthy that of the three segments from the linkage tester stock that appear to affect the mutation rate of R^r: Cornell, two are associated with increased mutation frequency. These two mutation-favoring segments were present in every plant of the F_1, which nevertheless showed sharply reduced mutation rate of R^r: Cornell. Presumably, in the F_1 plants, their effect was over-ridden by other segments from the tester stock with mutation-reducing effect. Of these, only one happened to be included among those identified by linkage with a marker gene.

The possibility of extracting strains of very high mutation rate depends upon the extent to which these modifier effects are cumulative. When two mutation-favoring segments are brought together, their effect in combination will not necessarily be an augmented increase in the mutation rate. Possibly the modifiers may accomplish their effects in the same way, and the plant with either one may yield mutations at as high a rate as the plant with both. The data available are not extensive enough to show the mode of interaction, but some indication may be secured from this backcross, segregating for the three mutation-favoring segments marked by *Y*, *J*, and R^r. Since only about half of the population could be classified for *J*, the data are further reduced in comparisons involving this effect. The data for the various combinations are given in Table 3.

TABLE 3

INTERACTION OF HIGH-MUTATION SEGMENTS R^r, Y, AND J

FIRST BACKCROSS

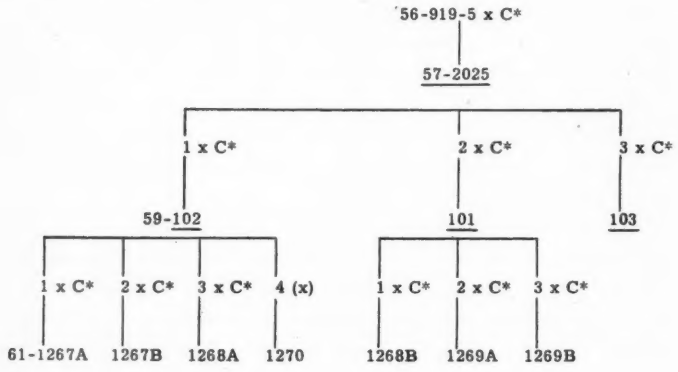
	No. plants	Mutation frequency	Mutation rate ($\times 10^{-4}$)	L. E.
R^r Y	66	21/12,819	16.4	25.1–10.1
R^r y	63	6/10,494	5.7	12.5– 2.1
R^g Y	46	1/ 4,825	2.1	11.5– 0.1
R^g y	49	2/ 4,319	4.6	16.7– 0.6
Total	224	30/32,457	9.2	132.1– 6.2
R^r J	41	15/ 8,609	17.4	28.7– 9.8
R^r j	21	1/ 3,384	3.0	16.5– 0.1
R^g J	26	1/ 2,695	3.7	20.7– 0.1
R^g j	31	0/ 3,367	0	10.9– 0
Total	119	17/18,055	9.4	150.8– 5.5
Y J	37	12/ 7,232	16.6	29.0– 8.6
Y j	26	1/ 3,825	2.6	14.6– 6.6
y J	30	4/ 4,072	9.8	25.2– 2.7
y j	26	0/ 2,926	0	12.6– 0
Total	119	17/18,055	9.4	150.8– 5.5

These data indicate that the effects of these three segments are cumulative. For example, among the $R^r R^r$ plants the presence of Y is distinctly favorable to mutation, and among $Y\ y$ plants the presence of R^r is distinctly favorable. Similarly, the addition of the mutation favoring segments in other combinations is in general favorable to mutation, so far as the scanty data permit the test to be made. Apparently, on the average, a plant of high mutation rate is increased in mutation frequency by the addition of a given segment in as great proportion as in a plant of low mutation rate.

The chromosome segment marked by the gene *a* could not be tested for relation to R^r mutation frequency, since the plants of the backcross progeny could not be classified for *A* constitution. The only indication of the relation of this segment to the mutation rate is the *A* constitution of the plants which yielded mutations, which was determined by a special progeny test of these plants. Presumably about half of the plants of the backcross progeny were *A A* and about half *A a*. If the *a* segment has a pronounced effect upon mutation frequency, the mutations should occur predominantly in plants of one or the other of these two classes. The test was made for 21 of the 22 plants that yielded mutations, the other plant being untestable because

the residue of viable seed was inadequate. Of the 21 plants, 7 were *A A* and 14 *A a*. The *A A* plants yielded a total of 12 mutations, the *A a* plants 17 mutations. The ratio of 12 : 17 in the contrasted classes would not be a significant deviation from equality, if the number of gametes tested in the two classes were equal. The excess of *a a* plants among the mutating individuals is suggestive of a possible relation, and it is interesting to note that the linkage suggested would be another case of an effect favorable to mutation in a chromosome segment from the tester parent.

Some further evidence on the relation of the segments marked by *Y-Pl*, *J*, and R^g was secured from progenies of the next generation. These included progenies produced from six plants of the first backcross which were backcrossed again to the Cornell stock, and from one which was self-pollinated. The relationship of the cultures studied is shown in Figure 1.



*Cornell stock .

FIG. 1. Relationship of cultures tested for mutation.

The seven plants of the first backcross that were used as parents of cultures 1267A–1270 were wholly unselected as to mutation rate and genotype. Mutation frequency was

determined in all these progenies. The mutation frequency in the second backcross cultures is shown in Table 4.

TABLE 4

MUTATION FREQUENCY OF R^r:CORNELL IN RELATION TO LINKAGE-TESTER GENES
SECOND BACKCROSS CULTURES

Culture	Genotype of Parent			Mutation frequency	Mutation rate	L. E.
	Y-Pl	J	R		(× 10-4)	
61–1267A	y-Pl/y-pl	j j	R^r R^g	7/8,538	8.2	16.9–3.3
1267B	Y-Pl/y-pl	j j	R^r R^r	2/3,388	5.9	21.3–0.7
1268A	y-pl/y-pl	j j	R^r R^g	0/2,820	0	13.1–0
1268B	Y-Pl/y-pl	j j	R^r R^r	2/3,147	6.4	22.9–0.8
1269A	y-pl/y-pl	j j	R^r R^g	2/2,826	7.1	25.6–0.9
1269B	y-Pl/y-pl	J j	R^r R^r	3/5,210	5.8	16.8–1.2

All the second backcross progenies were rather low in mutation rate, and none yielded a large enough number of mutations to provide significant confirmatory tests. Culture 1267A, which yielded 7 mutations, was the progeny of a plant of genotype R^r R^g, and gives some slight confirmatory evidence for the R relation. Of the 7 mutations, 6 occurred in R^rR^r plants and only 1 in an R^r R^g plant. In the other culture of this genotype which yielded mutants, 1269A, both of the mutants occurred in R^r R^r plants. The totalled mutation frequencies for R^r gametes in these two cultures were 8/6511 in R^r R^r plants and 1/4853 in R^r R^g plants. This is in agreement with the results on the R relation in the first backcross, and although the numbers are too small to be significant in themselves, they have considerable weight as confirmatory evidence.

No other data bearing on the R relation in the cross are at hand, since the self-progeny still to be discussed was produced by an R^r R^r plant. There are, however, comparable data from a cross of the Cornell stock with another R^g tester. The F_1 of this cross (reported in Table 3 of the preceding paper) yielded r mutations with a frequency of 9/79,858, including 6 r^r, 2 r^g, and 1 undetermined. The indicated mutation rate for R^r:Cornell gametes was therefore 1.7 (6.75/39,929), a pronounced reduction from the rate characteristic of the Cornell stock. Backcross progenies produced by crossing three F_1 plants with Cornell stock,

yielded 22 r^r mutations and 1 r^g mutation. The frequency of r^r mutations among R^r gametes tested was 20/15,036 in the $R^r R^r$ plants and 2/6,725 in the $R^r R^g$ plants. The difference in mutation rate is significant at the P = .05 level. The result is in agreement with that secured from the cross with the multiple linkage tester stock, in showing a substantial decrease in the seed-color mutation rate of R^r: Cornell in plants heterozygous for this chromosome segment, as compared to their homozygous sibs.

The plant which was selfed to produce culture 61–1270 was an $R^r R^r$ plant heterozygous for *Y-Pl/y-pl* and *J j*. This culture included 96 plants, bearing 34,344 seeds, and yielded 52 mutations (rate 15.1). The plants were classified phenotypically for *Pl* and *J*. The mutation frequencies determined for plants of these phenotypic classes are shown in Table 5.

TABLE 5

MUTATION FREQUENCY OF Rr:CORNELL IN RELATION TO LINKAGE-TESTER GENES

Culture 61-1270 (Self of an $R^r R^r$ *Y-Pl y-pl J j* Plant of First Backcross)

Phenotype	No. plants	Mutation frequency	Mutation rate ($\times 10^{-4}$)	L. E.
Pl	68	44/26,285	16.7	22.5–12.2
pl	28	8/ 8,059	9.9	19.5– 4.3
J	77	42/26,099	16.1	21.8–11.6
j	19	10/ 8,245	12.1	22.3– 5.8

The data from a self-progeny are not directly comparable with those from a backcross progeny. In a backcross progeny the comparative mutation rate in *Y y* and *y y* plants, for example, measures the effect of the genes of the *Y* segment plus those of the *y* segment, as compared to two doses of the genes of the *y* segment. If the genes modifying mutation rate are strongly dominant, the *Y* segment may show a significant effect in this comparison, even though the opposite segment carries modifiers of equal effect on mutation rate. For example, suppose that the *Y* segment carries a dominant modifier *M* which doubles the mutation rate, while the *y* segment carries at another locus a dominant modifier *N* of similar effect. In the backcross progeny,

the comparison of *Y M n/y m N* versus *y m N/y m N* plants (neglecting crossovers) would show the increased mutation rate due to *M*, superimposed upon the rate resulting from the action of *N*, which would be effective in both classes. But in the self-progeny, about ⅓ of the *Y* plants would be *Y M n/Y M n*, and would show a depressed rate due to the absence of *N*. Similarly, with modifiers of weak dominance, the results in backcross and self-progenies would not be comparable, due to the different proportions of plants with single and double doses of the genes concerned. Another differential factor concerns the reduction in detectable effect on mutation, due to crossing over between the marker gene and the modifier gene. This would be expected to be greater in the self-progeny than in the backcross progeny.

The data of Table 5 show a material difference in mutation rate associated with *Pl*, which is in the expected direction. It is not significant at the P = .05 level, but taken in connection with the results for the preceding generation it strongly indicates the validity of the linkage. The *Pl* relation is further discussed below.

The difference in mutation rate associated with *J*, as indicated in Table 5, is wholly insignificant. With equal mutation rate in *J* and *j* plants, the number of mutants expected in the *j* class would be only 12.5, and the total of 10 mutants observed is in reasonably close agreement. Thus the indication of a strong mutation-favoring effect of the *J* segment, shown by the first backcross generation, is not confirmed in this self-progeny.

Does this contradict the earlier indication of a *J* relation? Aside from the possibility that the *J j* plant which was the parent of culture 1270 may have been a crossover, there is reason to withhold a decision on this point. Among the 19 *j* plants of culture 1270 which in total showed a mutation frequency of 10/8,245, there was included one plant with a mutation frequency of 5/660. If this plant were considered a crossover, and were arbitrarily shifted to the *J* class, the mutation rates for the *J* and *j* classes would be 17.6 and 6.6 respectively, and the difference would be significant at the

P = .05 level. There is of course no basis for making this arbitrary reclassification. But the fact that it would have the effect stated emphasizes the importance of plant variability in these comparisons, and shows that the indication of equal mutation rate in the two classes is quite insecure. Even though the high mutation rate of the plant in question were the result, not of crossing over of *j*, but of the over-riding effect of some unusual combination of mutation modifiers, this reservation would still apply. In view of the high sampling error from plant variability, the original indication of the *J* relation in the first backcross also must be considered tentative.

It would be desirable, if possible, to exclude from the comparison the homozygous dominant plants, both for *Pl* and for *J*, in order to avoid in part the complications discussed above. This is not feasible for *J*, which is identified by a mature plant character, but it may be accomplished with *Pl*, by using as a basis of classification the seedling root-color effect. When the seeds are germinated in darkness, the roots of *Pl* (R^r) plants develop anthocyanin while the roots of *pl* (R^r) plants do not. This classification is often unsatisfactory because of poor root coloration, but in this material it gave clear separations.

Since all the plants of the culture were pollinated by a *y y pl pl* pollen stock, it was possible to classify the plants as *Y Y*, *Y y*, and *y y* by seed color and as *Pl Pl*, *Pl pl*, and *pl pl* by root color. This classification was made for all plants which yielded 200 seeds or more. Of the 96 plants, 70 produced 200 seeds or more, and the total was 31,031 seeds, including 46 mutants (mutation rate 14.9). The distribution of the mutants with relation to homozygous and heterozygous *Y* and *Pl* is shown in Table 6.

The result clearly shows that the increase in mutation rate associated with both *Pl* and *Y* is reduced in apparent effect in the self-progeny by the inclusion of the homozygotes *Pl Pl* and *Y Y*. The comparative mutation rates for *Pl* and *pl* plants in the 70 plants are 16.1 and 10.3 respectively, but when the 18 *Pl Pl* plants are excluded, the rates

TABLE 6

MUTATION FREQUENCY OF Rr:CORNELL IN RELATION TO HOMOZYGOSITY OF Y-Pl SEGMENT

Culture 61-1270 (Self of an *Rr Rr Y-Pl y pl J j* Plant of First Backcross)

	Pl Pl		*Pl pl*		*pl pl*		Total			
	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	No. plants	Mutation frequency	Mutation rate	L. E.
									(× 10⁻⁴)	
Y Y	8	1/3,625	5	4/ 2,260	2	0/ 430	15	5/ 6,315	7.9	18.5– 2.6
Y y	6	6/2,505	18	22/ 8,350	4	3/2,090	28	31/12,945	24.0	34.1–16.2
y y	4	1/1,840	14	5/ 5,665	9	4/4,266	27	10/11,771	8.5	15.6– 4.1
Total	18	8/7,970	37	31/16,275	15	7/6,786	70	46/31,031	14.8	19.8–10.8
Mutation rate (× 10⁻⁴)		10.0		19.0		10.3		14.8		
L. E.		19.8–4.3		27.1–12.9		21.2–4.1		19.8–10.8		

compared are 19.0 and 10.3. The corresponding change for the Y relation is from 18.7 : 8.5 to 24.0 : 8.5.

Considering only the data from plants not *Y Y* or *Pl Pl* (that is, the cells of columns *Pl pl* and *pl pl*, rows *Y y* and *y y* of Table 6) the results are roughly comparable to those of the first backcross, as shown in Table 1. The data are as follows:

	Mutation frequency	Mutation rate
Y y	25/10,440	23.9
y y	9/ 9,931	9.1
Pl pl	27/14,015	19.3
pl pl	7/ 6,356	11.0

The difference between the *Y y* and *y y* classes is significant at the P = 0.05 level: that between the *Pl pl* and *pl pl* classes is not. The agreement between this and the preceding progeny strongly indicates the significance of the linkage for both markers.

As in the preceding generation, the linkage is closer with *Y* than with *Pl*. The relation of mutation frequency to *Y* and *Pl* combinations, comparable to the first backcross data of Table 2, is as shown below.

As before, *Pl* fails to show any favorable effect upon mutation in the *y Pl* crossovers, indicating that the mutation-modifying gene or genes are closer to *Y* than to *Pl*. There is again a slight indication favoring the order *Y-M-Pl*

	Mutation frequency	Mutation rate
Y Pl/y pl	22/8,350	26.3
Y pl/y pl	3/2,090	14.4
y Pl/y pl	5/5,665	8.8
y pl/y pl	4/4,266	9.4

over *M-Y-Pl*, since crossing over between *Y* and *Pl* reduces the mutation-favoring effect of *Y*.

The reduced mutation rate in plants homozygous for *Y Y* or *Pl Pl* suggests the possibility of a dominant mutation-favoring modifier in the *y-pl* segment from the Cornell stock. Assuming a single dominant gene to be responsible for the increase in mutation rate of *Y Pl/y pl* plants over *Y Pl/Y Pl* plants, the data of Table 6 may be used similarly to indicate its effect and locus. Disregarding crossovers, this modifier (here designated *N*) is present as *N N* in the class *y pl/y pl*, *N n* in the class *Y Pl/y pl*, and *n n* in the class *Y Pl/Y Pl*. Corresponding data for the effect of the heterozygote compared to the homozygous recessive are therefore given by the cells of columns *Pl Pl* and *Pl pl*, rows *Y Y* and *Y y* of Table 6. These show the following relations:

	Mutation frequency	Mutation rate
Y y	28/10,855	25.8
Y Y	5/ 5,885	8.5
Pl pl	26/10,610	24.5
Pl Pl	7/ 6,130	11.4

The difference in mutation rate between *Y y* and *Y Y* plants is significant at the 0.05 level, and the difference between *Pl pl* and *Pl Pl* is just short of significance. This linkage also is closer with *y* than with *pl*.

The relation to *y* and *pl* recombinations is as follows:

	Mutation frequency	Mutation rate
y pl/Y Pl	22/8,350	26.3
y Pl/Y Pl	6/2,505	24.0
Y pl/Y Pl	4/2,260	17.7
Y Pl/Y Pl	1/3,625	2.8

The effect of *y* upon mutation rate is substantially unaffected by crossing over between *y* and *pl*, indicating the

order *N-y-pl*, or the order *y-N-pl* with *N* much closer to *y* than to *pl*. The effect of *pl* is possibly reduced in *Y pl* crossovers, as compared to *y pl* non-crossovers, but is not wholly lost, as might be expected if the order is *N-y-pl*.

Obviously these indications of the locus of *M* and *N* with reference to *Y* and *Pl* are very questionable, because of the small numbers involved in the comparisons. It is probable that both modifiers are closer to the *Y* locus than to the *Pl* locus, and possible that both are located between *Y* and *Pl*. The considerations suggesting their loci are based on the assumption that the effects of the chromosome segments indicated are the result of single factors in each case, and that these factors are dominant in effect to such a degree that the effect of *M m*, for example, is substantially equal to the effect of *M M*. The possibility that *M* and *N* are allelic is not excluded by the evidence, but in this case the effect on mutation rate must be substantially greater for the heterozygote *M N* than for either homozygote, *M M* or *N N*.

Discussion

The results indicate that the frequency of the gene mutation studied, seed color mutation of R^r: Cornell, is susceptible to a surprisingly high degree to the action of modifying genes. The marker-genes of the linkage tester stock used covered effectively only a relatively small part of the total genotype; the populations tested were large enough to identify only modifiers of rather strong effect; and the experimental design was such as to detect only those modifiers in which the allele derived from the tester stock was dominant to the allele derived from the Cornell stock. Yet three of the seven marked segments proved to have detectable effects on mutation frequency. Presumably various other modifiers of similar action were segregating in the backcross tested, but were indistinguishable because they were unlinked, or were not closely enough linked, with the marker genes used. The presence of additional low mutation modifiers is indicated by the extreme reduction in

mutation frequency in the F_1, despite the presence in all the plants of the high-mutation modifiers demonstrated in the backcross generation. Presumably also there is a corresponding class of modifiers in which the allele present in the Cornell stock is dominant to that present in the tester stock. These would be identifiable in the converse backcross, $F_1 \times$ tester stock, though it is unlikely that the mutation rate in this cross would be high enough to make their identification feasible.

These alleles are referred to as dominants on the basis of the comparison of the heterozygote with only one of the two homozygotes. They would not necessarily appear as dominants if the effect of the other homozygote were known. For example, the gene *Dt*, which has a very pronounced effect on the frequency of the mutation a → A, shows disproportionately increasing effects with increasing gene dosage. The number of mutations produced in endosperm tissue by the *Dt Dt Dt*, *Dt Dt dt*, and *Dt dt dt* genotypes, respectively, are in the approximate ratio 17:3:1 (Rhoades, 1941). Now if a gene of comparable action on R → r mutation, here designated *D*, were present in the Cornell stock, and its allele *d*, comparable to *dt*, were present in the tester stock, the backcross ($F_1 \times$ Cornell stock) would include plants of genotype *D D* and *D d*. The mutation rate would be lowered in the *D d* class, and the gene *d* would appear as a dominant modifier reducing mutation frequency.

In the case of the R^g segment, the possibility must be considered that the reduction in mutation frequency may be brought about by some direct interaction of the R^g and the R^r gene rather than by a modifier linked to R^g. The possibility that mutation of *R* may be associated with crossing over at or near this locus is not excluded, although it has been shown (Stadler, 1948) that the mutation is not associated with crossing over between *R* and *G*. Further data on the relation of *R* mutation to crossing over will be submitted shortly. Proof that the effect is due to a modifier rather than to R^g requires the demonstration of a crossover separating the two genes.

The extent of the increase or decrease in mutation frequency due to the action of the individual modifiers can be estimated only roughly. The ratios of mutation frequency in the contrasted classes in the backcross progeny were 1.0 : 2.3 for the *Y* segment, 3.5 : 1.0 for the R^g segment, and 1.0 : 9.5 for the *J* segment. But these ratios are based upon a total of only 30 mutants and are subject to high sampling errors. The evidence on the extent of the *J* effect is especially weak, since it is based upon only 17 mutants, 16 of which occurred in *J j* plants and only 1 in a *j j* plant. The observed yield of 1 mutant in the *j j* class does not significantly contradict a frequency of nearly 5 mutants in this class. Although the difference in mutation rate between the *J j* and *j j* classes is clearly significant (assuming the classes to be comparable) the result does not contradict the possibility of an increase in mutation rate as low as 3-fold for the *J* segment. The 2.3-fold increase found for the *Y* segment is in good agreement with the increase indicated for this segment in the self-progeny (Table 6), and the reduction in mutation rate associated with the R^g segment in the backcross progeny (3.5 : 1.0) is fully matched by the reduction associated with the R^g segment from the McBaine stock (4.4 : 1.0). In view of the indication that modifying factors may be very numerous, the differences in mutation rate associated with specific segments are not necessarily to be ascribed to the effects of single modifier genes. Detailed studies of the individual segments will be necessary to determine whether separable genes are involved and to indicate the loci of the modifiers. The 3- to 4-fold modification of the mutation rates indicated for the modifiers would be substantially increased by evidence of frequent crossing over between modifier and marker.

The indication that gene mutation is so readily affected by diverse modifiers encourages the hope that the mutation process may be subject to experimental control, for each modifier must produce its effect by altering in some way conditions which affect the probability of mutation. It is plausible to suppose that the occurrence of gene mutation

is determined by chemical conditions within the cell. But even if the necessary conditions were precisely known, it would probably be impossible in most cases to approximate them by chemical treatments, because of the insulating mechanisms of the living organism. Chemical alterations effected by gene action escape this limitation.

If the indication of large numbers of modifiers with pronounced effects on mutation frequency is substantiated by further investigation, these modifiers may be very useful in the study of the nature of gene mutation. Two approaches may be mentioned:

(1) The extraction of strains in which specific gene mutations are made frequent enough to permit direct experimental study of the phenomenon.

A major obstacle to the experimental study of the mutation process has always been the very low frequency of mutation. The study of X-ray effects on mutation was originally motivated chiefly by the hope that gene mutation might thus be made frequent enough for effective experimental investigation. This hope has not been realized. The frequency of point mutations was greatly increased by the treatment, but critical study of the mutations induced has shown that various extra-genic alterations may closely simulate gene mutation. There are no general criteria by which gene mutations may be distinguished from other Mendelizing variations among a group of induced point mutations at miscellaneous loci. Consequently, the high general mutation rate secured by the use of X-ray treatment does not provide suitable experimental material for the study of the reaction in which a gene is converted to an alternative form. Further, the recognition of the variety of extra-genic phenomena simulating gene mutation makes it seem doubtful that the study of this reaction could be made effectively by means of any experiments on the general mutation rate, for spontaneous mutations also may include extra-genic alterations. The investigation of gene mutation seems to require the intensive study of the mutation of specific genes especially suitable for critical analysis.

This of course intensifies the difficulty arising from the rarity of the mutations.

The results here reported suggest that it may be possible to bring the frequency of occurrence of the specific mutation here studied to a surprisingly high level, by combining favorable modifier genes. The Cornell stock itself represents an unusual combination of modifiers favorable to the $R^r \rightarrow r^r$ mutation, and the results indicate that its high mutation rate is due to this fact rather than to any inherent instability of the R^r: Cornell gene. The further increase in mutation frequency resulting from the addition of the *Y*-linked and *J*-linked modifiers, if maintained proportionately at high mutation levels, and if cumulative in effect, might make possible a considerable multiplication of the characteristic Cornell rate. This might be accomplished by repeated backcrossing to the Cornell stock, with retention of the *Y* and *J* segments. In some cases, as appears to be probable for the *Y* segment, the substitution of a segment carrying a mutation-favoring modifier might fail to increase mutation rate because the Cornell stock may carry an equally favorable modifier in the corresponding segment.

It should be possible to make use of unmarked segments favorable to mutation, as well as the marked segments, by selection based upon direct mutation rate determinations in the individual segregating plants. Using mutation frequency in male rather than in female gametes, it is feasible to make significant mutation rate determinations for individual plants. If mutation modifiers are as frequent as these results suggest, continued backcrossing with selection based on individual tests should quickly isolate strains of greatly increased mutation rate.

(2) The use of the various mutation modifiers as differential reagents, so to speak, in the analysis of gene differences.

The known alleles of *R* include several groups of distinctive action, and some of these are known to mutate in distinctive ways. In the r^{ch} group, in which much increased pigmentation of the pericarp and certain other tissues is

superimposed upon the usual plant-color pattern, the two alleles studied yield plant-color mutations in which some of the pericarp-color and plant-color effect is retained. On the contrary, in the various alleles of the Cornell group, plant-color mutations have in all cases resulted in complete loss of plant-color, including the slight pericarp-color effect which occurs in this group. In the Cornell group, plant-color mutations occur independently of seed-color mutations. But in certain alleles of the Winnebago group, which is characterized by dilute plant-color effect, there is limited evidence suggesting that complete loss of seed-color and plant-color effect occurs simultaneously in a single mutational step.

Previous studies, as noted in the preceding paper, have indicated that the modifier complexes affecting the mutation rates of different R^r alleles are different, and that the modifier complex affecting the seed-color mutation rate of R^r: Cornell is different from that affecting the plant-color mutation rate of the same allele. The occurrence of a mutation for plant-color in R^r: Cornell greatly reduces the subsequent frequency of mutation for seed color (Stadler, 1946 and unpub.), but whether or not this reduction in seed-color mutation rate involves a change in its relations to mutation modifiers is unknown.

A priori it may be supposed that a group of mutation modifiers identified by their effects upon the frequency of a given mutation may include modifiers of various degrees of specificity, ranging perhaps from modifiers which may affect mutation frequency in general to modifiers which may affect the frequency of only a single type of mutation of a single allele. The seed-color mutation of R^r: Cornell may thus serve as a convenient detector for the identification of a collection of mutation modifiers, among which there may be many which affect various other mutations at the R locus and perhaps at other loci. The testing of an adequate sample of such modifiers upon the various alleles of R in their various types of mutation might yield evidence of systematic relations in the response of these

alleles to the conditions affected by the various modifiers. This would provide a basis for analytical investigation of the mutation process.

Summary

1. The frequency of seed-color mutation of R^r: Cornell, which occurs at the rate of 18.2×10^{-4} in the Cornell stock, was reduced to 0 in the F_1 of a cross with a multiple linkage tester stock. The number of R^r: Cornell gametes tested for mutation in the F_1 was sufficient to have yielded 22 mutations at the rate characteristic of the Cornell stock.

2. The backcross, $F_1 \times$ Cornell stock, yielded 30 mutations (rate 9.2×10^{-4}). The incidence of these mutations in relation to the segregation for the various marker genes indicates the presence of dominant genes favoring seed-color mutation of R^r: Cornell in the Y-marked segment and the J-marked segment of the tester stock. Similarly, the R^g-marked segment shows an effect unfavorable to the occurrence of the mutation.

3. The increase in mutation rate associated with the presence of the J segment appears to be highly significant statistically ($P < .01$). Since the statistical comparison assumes that sampling error due to individual plant variability is negligible, and since the results of the experiment as a whole indicate that plant variability may be very great, the evidence for the occurrence of this modifier is not considered conclusive.

4. The increase in mutation rate associated with the Y-segment was not quite significant in the backcross generation, but was clearly confirmed in the self-progeny of a backcross plant. The ratio of mutation rate in the $y\ y$ vs. the $Y\ y$ class was 1.0 : 2.3 in the backcross generation and 1.0 : 3.5 in the following generation.

5. The decrease in mutation rate associated with the R^g segment was significant in the backcross generation ($P < .05$) and was supported, in numbers inadequate for significance, in the second backcross generation. The location of a modifier in this segment is supported also by sig-

nificant evidence of the same sort from a cross of Cornell with another stock. The ratio of mutation rate in the $R^r R^r$ vs. the $R^r R^g$ class was 3.5 : 1.0 in the backcross generation of the cross with the linkage tester stock, and 4.4 : 1.0 in the backcross generation of the other cross mentioned.

6. The effect of the modifiers upon mutation frequency appears to be cumulative, so far as may be judged from the limited data.

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THE IMPORTANCE OF PLASMOLYSIS AND OSMOTIC PRESSURE IN MAKING NATURAL MOUNTS OF *SPIROGYRA* AND *CLADOPHORA*

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THE word "plasmolysis" is derived from the Greek *plasm* having biological reference to living matter of a cell, and *lysis* signifying a loosening or detachment. Specifically, it refers to the contraction or shrinkage of the protoplasm of a cell due to loss of water by exosmosis. Plasmolysis occurs when a living plant cell is placed in a sugar or salt solution in which the concentration of the solute is greater than that within the cell. The volume of the cell decreases slightly at first, after which the protoplasm (including the chloroplasts) draws away from the cell wall and contracts. If such a cell is then placed in water, the protoplasm gradually regains its original form.

This plasmolytic action can be explained in terms of *osmotic pressure,* which develops as a result of the flow of solvent toward the solution having a higher molecular concentration. The osmotic pressure of the solution is then greater than that of the cell sap. Plant cells usually contain one or more vacuoles. Normally, the osmotic pressure of the solution within the vacuole is such that water tends to enter the cell. When a cell is immersed in a solution having the same osmotic pressure as the cell sap, that solution is said to be isosmotic or *isotonic* with the cell sap. An example of this is the pond water in which fresh-water algae live. A *hypotonic* solution is one whose osmotic pressure is lower than that of the vacuolar sap, whereas a *hypertonic* solution has a pressure higher than that of the sap.

The osmotic pressure increases with the concentration and temperature of the solution. Crystalloids produce a higher osmotic pressure than do colloids. The nature of the separating membrane also determines the extent of osmosis. Since most cells in water absorb the latter, the protoplasm presses against the elastic cell wall and stretches it. When the pressure admitting water into the cell is balanced by the inward pressure of the stretched cell wall, the cell is said to be turgid or in a state of turgor.

When *Spirogyra* or *Cladophora* cells are placed in a solution other than pond water, the osmotic pressure of the solution tends to force water out of the cells. The hydrostatic pressure of the cell sap against the cell wall is known as *turgor pressure*. The inward pressure of the stretched cell wall is equal and opposite to the turgor pressure. When *Spirogyra* is in its natural habitat, its turgor pressure is at a maximum. However, when plasmolysis occurs, the turgor pressure is at a minimum. The osmotic pressure of the surrounding solution is then said to be greater than the osmotic pressure of the cell sap minus the turgor pressure.

Every substance absorbed by a plant cell must pass through two membranes, the cell wall and the plasma membrane. The former is permeable to most dissolved substances. The plasma membrane, or outermost limit of the protoplasm, is semi-permeable in nature, admitting only certain substances. The term is used for convenience, for in most plants no definite structural plasma membrane can be seen under the microscope. In the measurement of osmotic pressure of plant cells collodion membranes, precipitation membranes, parchment paper, and animal bladders have been employed. The latter have osmotic properties similar to that of plant cell walls, while precipitation membranes (usually copper ferrocyanide) give rise to high osmotic properties and are only slightly permeable to many substances. In any event, the membrane acts as a sieve, allowing passage of molecules of the diffusing substances.

A membrane acts as a sieve or " ultrafilter " when it is a porous, heterogeneous substance. The molecules of the so-

lute may then pass only through the pores. If, however, the membrane is homogeneous, the dissolved substance passes through it in solution or is adsorbed at the surface of the membrane. The permeability of the plasma membrane varies considerably during the life of the cell due to changes in external conditions. The higher the temperature, the greater the permeability, up to a certain point. Visible light, ultraviolet rays, radium, and X-rays increase permeability. Hydroxyl groups lower penetrating power, while ethyl and methyl groups increase it.

Some substances enter plant cells very slowly or not at all, while others are readily absorbed. Hydrocarbons and fat solvents pass readily through the plasma membrane, whereas sugars and salts pass more slowly. Weak acids and bases penetrate rapidly, while strong electrolytes penetrate slowly. The more concentrated the external solution, the less water the cell can absorb. The ions of a dissolved substance must also be considered. Sodium and potassium ions increase cell permeability; bivalent cations decrease permeability. Recently sodium, potassium, chlorine, calcium, and other elements have been made radioactive, and their penetration into living protoplasm can be traced by means of the Geiger counter. Atomic research has thus made a significant contribution toward a better understanding of plasmolysis and permeability.

Having thus reviewed the factors involved in plasmolysis and osmosis, their application in attempts to mount *Spirogyra* and *Cladophora* in the natural state will be made more comprehensible.

Spirogyra

Spirogyra is an unbranched, filamentous, colonial green alga occurring in large masses in fresh-water ponds and pools. It may be macroscopically distinguished from most other filamentous green algae by its slippery feeling due to the presence of a gelatinous sheath outside the cell wall. Microscopically the cells are cylindrical and attached end to end. The diameter of the cell varies from 25 to 30 mi-

crons, while its length is from 200 to 300 microns. The outer wall is about 3 microns thick; in growth it increases in length, but not in diameter.

A thin layer of dense cytoplasm, not over 10 microns thick, lies just inside the wall of each cell, and is held against the wall by the hydrostatic pressure of the cell sap in the central vacuole.

In each cell, characterizing this genus, there is a green, spirally coiled, ribbon-like chloroplast from 5 to 10 microns in thickness. The edges may be smooth, serrated or crenated. The chloroplast does not lie in the center of the vacuole, but is attached to the outer portion of the peripheral cytoplasm, and its coils follow the cylindrical form of the cell. Its specific gravity and refractive index are greater than that of the vacuolar sap. In some species there are two chloroplasts in each cell. Spaced at regular intervals along each chloroplast are small, spherical bodies known as pyrenoids, which function as condensation centers during starch formation. They are connected by a central strand extending along the length of the chloroplast. The chloroplasts of adjacent cells are not connected. They are separated by a common cellular end wall consisting of a central layer of pectose having a cellulose layer on each side of it.

The nucleus, which is often invisible in the living cell, is suspended in the center of the vacuole by thin, dense cytoplasmic strands, each of which extends to the peripheral cytoplasm and terminates immediately beneath a pyrenoid.

The writer considered that it would be useful for college departments of biology to have on hand permanent slides of *Spirogyra* exactly as it appears in the living condition, retaining the shape and natural color of the chloroplasts with no plasmolysis induced. This was easier said than done, for this plant was found to be extremely sensitive to most liquids other than pond water, tap water or distilled water.

The two most important problems, therefore, were (1) to fix the spiral chloroplasts in place, and (2) to retain the

chlorophyll and consequently the green color. These factors had to be kept in mind in the selection of a mounting medium. Fixation in alcohol, no matter what the concentration, was found to be useless, since it extracted the chlorophyll and also induced plasmolysis. Therefore alcoholic mounting media, such as euparal, diaphane, and Venetian turpentine, were immediately ruled out, although they were tried just to be sure of their effects.

When plasmolysis occurs in *Spirogyra,* the chloroplast becomes detached or loosened from the peripheral cytoplasm and contracts into a rod-shaped or irregularly rounded mass in the center of the vacuole which loses considerable water. The peripheral cytoplasm itself does not usually withdraw from its proximity to the cell wall; however, with more concentrated solutions it does tend to contract, and a definite space can be seen between the plasma membrane and the cell wall.

Various other fixing agents were tried in different concentrations. Formaldehyde did not extract the chlorophyll, but caused plasmolysis. Osmic and picric acid in various dilutions were found to be useless, for, although they fixed the chloroplasts in place, they removed the natural green color. Dilute acetic acid also caused plasmolysis. At the time that these failures were occurring, other mounting media were considered. In order to mount in Canada balsam, the plant would have to be placed into xylol. To mount in a synthetic resin such as Permount, the *Spirogyra* filaments would have to be placed first into toluene. Unfortunately, both xylol and toluene were found to induce twisting of the filaments as well as marked plasmolysis.

It soon became clear that *Spirogyra* would have to be mounted in a water-soluble medium, since the plant could not tolerate any other medium. Filaments were transferred from pond water to pure glycerine, which is soluble in water. Plasmolysis occurred, but the color was retained. Very dilute glycerine was then used, but plasmolysis occurred immediately. The chemical composition of the chloroplasts

was not known, but it was assumed that proteins were present in large percentage. Therefore anything that would fix proteins would probably be the substance or method sought.

It occurred to the writer that heat, if applied suddenly, would tend to fix or coagulate the proteins. A few *Spirogyra* filaments were placed on a slide in a drop of pond water, and the slide was then placed upon a hot aluminum plate. The water evaporated almost immediately, and the chloroplasts remained in spiral form, but they seemed to have lost some color and to have become slightly distorted. After numerous other attempts, success was obtained with hot water. Thus only one method was found to be successful in making permanent slides of *Spirogyra* as it occurs in the natural condition. This method is outlined as follows:

(1) Heat a half-filled beaker of pond water until boiling begins.

(2) Remove from the source of heat and wait until the bubbling subsides.

(3) Drop a small mass of filaments into the water, and allow them to remain for at least 30 minutes.

(4) Transfer to 10 per cent glycerine for 30 minutes, then transfer to 30 per cent, 50 per cent, and 75 per cent glycerine also for 30 minutes each.

(5) Allow the 75 per cent glycerine to evaporate until the liquid is of the consistency of pure glycerine.

(6) Place a drop of the latter on a clean slide. Into it place several small *Spirogyra* filaments, then apply a cover slip. Just enough glycerine should be used as to prevent seepage beyond the edges of the cover slip.

(7) Ring with melted glycerine jelly. (Soak 4 grams gelatin in 30 cc. water, add 30 cc. glycerine, heat, and add a very small crystal of phenol or camphor. Heat to melt before ringing cover slips.)

(8) Ring with gold size (used for affixing gold leaf to windows), and repeat with another coat after the first dries.

(9) Apply a final ring of synthetic resin mounting medium, such as Permount, or use Bell's microscopic cement if available.

Such slides made by the writer one year ago are still in excellent condition. The *Spirogyra* chloroplasts have retained their natural green color and shape, no plasmolysis having occurred. The chief disadvantage of such slides is that the mounting medium, being glycerine, is in liquid form, and may be forced out when excessive pressure is exerted upon the cover slip in using the high power objec-

tive carelessly. However, they have served a most useful purpose, and are expected to last for a long period of time.

Cladophora

Cladophora is a branched, filamentous, rough green alga usually found growing attached to objects in shallow fresh water, especially along shores of lakes. Some species, however, are brackish or marine. The diameter of the filaments is greater at the base than at the ends. The cylindrical cells are united end to end. Just inside the thick cell wall is a cylindrical layer of dense, net-like cytoplasm in which are embedded hundreds of small, round or oval chloroplasts. Numerous pyrenoids are usually present. Many nuclei are embedded in the innermost portion of the peripheral cytoplasm beneath the chloroplasts. The large central vacuole is filled with cell sap. In addition there are smaller, regularly spaced peripheral vacuoles, 4 to 24 microns in diameter, in which are stainable, plate-like crystalloids never larger than 8 microns. By macerating filaments in a weak neutral-red solution, these vacuoles and their contained bodies stand out. Zoospore and gamete formation are frequently observed.

The methods described above for *Spirogyra* were tried with *Cladophora,* but plasmolysis occurred more markedly than in the former plant. It was found that *Cladophora* is much more sensitive than *Spirogyra* and plasmolyzed very quickly. When dropped into boiling water, a marked degree of shrinkage occurred, therefore this method of fixation, although used successfully with *Spirogyra,* was considered to be useless in the case of the other plant.

Only one method gave temporary results. Small branches of *Cladophora* were mounted directly from pond water into a 5 per cent gelatin solution on a slide, and a cover slip affixed. For three days the filaments remained natural in appearance, but on the fourth day plasmolysis was found to have occurred. Marked shrinkage had taken place in each cell, so that the green protoplasm in each cell was reduced to a small rectangle in the center of the cell.

It was concluded, after careful study of the results, that plasmolysis had taken place because of the growth of molds and bacterial colonies in the gelatin. However, when substances to prevent mold growth, such as phenol and camphor, were added to the gelatin when mounting the *Cladophora,* the fungicidal and bactericidal substances caused immediate plasmolysis.

Due to the extreme sensitivity of *Cladophora,* it is evident that much additional study and experimentation will be necessary before natural mounts of this green alga can be made without plasmolysis.

LONGEVITY OF THE PARASITIC WASP *HABROBRACON JUGLANDIS* ASHMEAD

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INTRODUCTORY HISTORY

AMONG animals, insects provide favorable material for a study of longevity and in recent years increasing attention has been focussed on this problem. Rau (1910), investigating the life span of the saturniid moth, *Samia cecropia,* found that the mean length of life of the male was about 11 days, whereas that of the female was only about 8 days. Rau and Rau (1912, 1914) reported that the life span in *Samia cecropia* was not significantly shortened or lengthened by mating, but that low temperatures greatly prolonged their life span.

Lutz (1915) studied the duration of life of *Drosophila melanogaster* in the presence of water, but in the absence of food. The results showed that water increased the mean length of life. Loeb and Northrop (1916), performing the same experiment, stated that the mean duration of life of the flies varied inversely as the temperature between 9 and 34° C. Loeb and Northrop (1917) found that there existed a definite temperature coefficient for the duration of life in *Drosophila* and that the nature of food influenced the duration of life. Northrop (1917) found that the total duration of life in *Drosophila* can be prolonged if the larval period is prolonged by inadequate feeding.

Although *Drosophila* has been a favorite insect for experimental study for many years, the first accurate report on its duration of life was made by Pearl and Parker in 1921. They made a series of reports on the factors influencing the duration of life. They reported that there were wide differences in the life span of different types of *Drosophila* and

that the basis of these differences was hereditary rather than environmental. This opened a new period in the history of investigations on longevity, particularly in entomology. Pearl and Parker (1922) found that females lived longer than males at all densities, when full-fed. On the contrary, Pearl and Parker (1924) observed that under starvation the mean duration of life was almost the same in the vestigial as in the wild-type.

Alpatov and Pearl (1929), studying longevity of *Drosophila* under different temperatures, observed that females were longer lived than the males in all series of experiments. Gonzalez (1923) attempted to localize the factors controlling the duration of life of *Drosophila.* He also studied the effects of food and temperature of its life span. Bowen (1936) found that the male outlived the female in *Trichogramma,* a parasite on the oriental fruit moth, and Menusan (1936) demonstrated that temperature and humidity have marked effects on duration and viability of different stages of bean weevil, *Bruchus obtectus.*

Lilleland (1938) used *Drosophila pseudoobscura* in experiments dealing with the duration of life in the absence of food and made comparisons at different temperatures, humidities, and population densities. Greiff (1940) investigated the duration of life of wild-type *Drosophila melanogaster* and its ebony mutant in the absence of food and water. He found that the mean duration of life of the ebony mutant exceeded that of the wild-type in a statistically significant manner. The mean length of life of the wild-type male was significantly greater than the mean length of life of the wild-type female, but the difference between the mean duration of life of the ebony female and that of the ebony male was non-significant.

Up to the present time the problem of longevity in *Habrobracon juglandis* (Ashmead) has been only occasionally and insufficiently attacked by other investigators. Doten (1911) worked with small samples of *Habrobracon* under normal laboratory conditions with temperatures ranging between 70° and 80° F. He reported that females

if fed on honey water lived an undetermined number of months, and that they outlived the males. If not fed at all the females died in from 11 to 15 days. Males when fed were kept alive for an indefinite time, but if not fed, they died in a week or ten days. Doten concluded by emphasizing the necessity of controlling the conditions under which the experiments are performed in order to draw valid conclusions.

Harries (1937) determined the approximate duration of the developmental stages of *Microbracon hebetor,* which is synonymous with *Habrobracon juglandis,* but he did not report any investigation of the duration of life of the imago. Extensive work has been done on *Habrobracon* by Dr. P. W. Whiting, of the University of Pennsylvania, and by his coworkers who have investigated many phases, but there is no evidence in literature that any of them have studied the life span of this insect.

In the following investigation on longevity of the parasitic wasp, *Habrobracon juglandis,* a comparative study was made of the mean duration of life of the wild-type and of the double mutant short-wing, white-eye type, which will be referred to in this report as the double mutant.

The writer is deeply indebted to Dr. E. S. McDonough, of the Department of Biology of Marquette University, under whose kind direction the present investigation was carried out, and wishes to express sincere appreciation for his advisory assistance and helpful criticism during the progress of this study; to Rev. R. H. Reis, S.J., head of the Biology Department of Marquette University, for help and encouragement; and to Dr. P. W. Whiting, of the University of Pennsylvania, for valuable criticism, literature and other help.

Materials and Methods

All the experiments in this investigation were carried out under constant environmental conditions. The cultures were incubated at about 30° C. which, according to Whiting (1921) and Maercks (1933), is the optimum tempera-

ture for *Habrobracon*. The humidity in the incubator was controlled by employing a saturated solution of ammonium chloride which has been reported by Obermiller (1924) and in the International Critical Tables (1926) to maintain a humidity of 79.5 per cent at 30° C. Water was added daily to the solution, or when necessary, to maintain a certain level which had been indicated on the outside of the beaker.

The two stocks of *Habrobracon* as well as the host caterpillars were obtained from Dr. P. W. Whiting, and the technique used in handling the insects was that described by Whiting (1921, 1937).

A pair of wasps was selected and placed with four full-grown host caterpillars in a shell vial 20 × 70 mm. and incubated. After mating, the female stings the caterpillars and sucks juice from the punctures. The males must be fed honey-water as they do not feed on caterpillars. After the caterpillar has become flaccid, the female deposits her eggs on it, usually on the under side. In about four days, tiny maggot-like larvae may be observed clinging to the integument of the host caterpillars. The adult wasps are now transferred to a new vial with fresh caterpillars. Growth of the *Habrobracon* larvae is rapid and soon white cocoons appear, and the host caterpillars are reduced to shrivelled remnants. In ten days from the time the pair was set, eclosion begins. From four to seven wasps were obtained from each host caterpillar.

As soon as the adults of *Habrobracon* emerged, they were transferred to shell vials. A population density of four males or four females per vial was maintained throughout the investigation. As was pointed out by Pearl and Parker (1922):

> Putting all data together we have here indisputable evidence that the density of population is a significant factor in influencing the duration of life.

Wasps that seemed abnormal or undernourished were not used for experimentation, but were discarded, and the experimental stock was not used for breeding, but was segregated before mating. The vials containing the test stock

were labeled and placed flat in racks in the incubator. All stock was fed honey-water every other day. This honey-water consisted of three parts of honey to two parts of water which was mixed and heated slightly to prevent fermentation and to insure the possibility that all insects received the same concentration of honey. Routine feeding was accomplished by using a small medicine dropper with which a small drop of food was placed in the top-arch of the vials which housed experimental *Habrobracon*. This, according to Doten (1911), reduced the entanglement of insects in honey-water. Losses due to entanglement were also cut down to a minimum by removing remains of old honey before administering a new droplet. This precaution also serves as a safeguard against mold and other contaminations.

Each morning at the same hour the test stock was examined and records were made of those that had died. Care was taken that no vials were out of the incubator for more than fifteen minutes during routine feeding and checking. To test the validity of results, breeding was continued until four or five runs of experiments had been made of both males and females of the two stocks, yielding 1,180 wild-type wasps, and 1,366 double mutant wasps. Thus a total of 2,546 wasps was tested.

Results

Table I presents the frequency distribution of males and females, respectively, of each of the two stocks of *Habrobracon*.[1] The class centers of the various groups are shown, together with the number of wasps that died in the respective five-day intervals. Table II gives the chief biometric constants[2] derived from this investigation.

The mean duration of life of the wild-type female was found to exceed that of the wild-type male by 5.157 days

[1] A daily record was kept of the number that died, but in order to conserve space only the frequency table is included here.

[2] The short method described in "Principles of Genetics," by Sinnott and Dunn, p. 140, was used in computing the various constants.

TABLE 1

FREQUENCY DISTRIBUTION OF 1,180 WILD-TYPE AND 1,366 DOUBLE MUTANT WASPS

Class center in days	Number of Habrobracon that died in the 5-day period			
	Wild-type		Double mutant	
	Males	Females	Males	Females
4	12	15	18	16
9	40	26	80	30
14	81	26	176	74
19	142	41	214	89
24	165	62	193	145
29	129	119	81	85
34	58	75	41	58
39	52	44	13	24
44	21	37	3	13
49	.....	24	.....	13
54	.....	11	.....	.....
	700	480	819	547
Total	1,180		1,366	

A daily record was kept of the number that died, but in order to conserve space only the frequency table is included here.

TABLE II

CHIEF BIOMETRIC CONSTANTS

	Mean duration of life (days)	Standard deviation (days)
Wild-type male	24.093 ± 0.334	8.825 ± 0.236
Wild-type female	29.250 ± 0.517	11.335 ± 0.366
Double mutant male	19.946 ± 0.259	7.410 ± 0.183
Double mutant female	23.991 ± 0.407	9.505 ± 0.288

The short method described in "Principles of Genetics," by Sinnott and Dunn, p. 140, was used in computing the various constants.

under the conditions of this experiment. By dividing this difference by its own standard error, it was found that this difference was statistically significant.

The mean duration of life of the double mutant female exceeded that of the double mutant male by 4.045 days. This difference was statistically significant.

The mean duration of life of the wild-type male exceeded that of the double mutant male by 4.147 days, and this difference was found to be statistically significant.

The mean duration of life of the wild-type female exceeded that of the double mutant female by 5.259 days, and this difference also was found to be statistically significant.

DISCUSSION OF RESULTS

In this investigation the mean duration of life of the wild-type wasp was significantly longer than that of the double mutant wasp. In endeavoring to explain this dif-

ference it is important to consider the part that food and environment play in the life of an animal.

It is a well known fact that, aside from temperature, an adequate food supply is essential to normal life, and that the nature of the food affects the life span. Pearl and Parker (1924) advanced the idea that the inherent potentiality of the organism in respect of longevity is determined by its individual, inborn physico-chemical organization, and that the realization or expression of the potentiality of longevity is a function of the environment, for this environment determines in considerable part the rate at which the vital resources are used up. In considering the physiology of animal metamorphosis, Kopec (1924) remarked:

> It seems to me to be unquestionable that the duration of life depends on the character of metabolism, in other words, that natural death is a function of metabolism.

This is further substantiated by Pearl and Parker (1924):

> Normally the living organ gains its energy for the conduct of life, and material for the repair, within limits, of the wastage of its tissues in the business of living, by the process of taking food. In other words the regular rewinding of the vital clock is accomplished by feeding. If the renewal or rewinding process is defective in any particular, the result will be to shorten life below what would have been attained under more perfect metabolic functioning.

The normal life span of an organism seems to be determined by the production of a substance leading to old age and natural death, or by the destruction of a substance which normally prevents old age and natural death. According to Ruzicka (1929) longevity also depends on the rate of gradual condensation of biocolloids which underlie the process of aging. He suggests:

> The gene of duration of life is not preformed but it arises by continual changes of biocolloids provoked by external influence and ends by condensation rendering metabolism impossible, thus causing death.

After considering the above, and keeping in mind that the technique used insured constant environmental conditions throughout all the experiments, it becomes evident that environmental factors are not the basis of the differ-

ence in longevity. Since both strains were reared at the same time, the difference noted is definitely believed to be hereditary. In these investigations the double mutant short wing, white-eye wasps were shorter lived. The mutant genes apparently affect the summation of physiological processes under the conditions of these experiments so that the mean length of life is shortened. It might be suggested then that the mutant genes have as a part of their somatic expression a reduced duration of life as compared with the wild-type, under the conditions of these investigations. Referring to the work done by Gonzalez (1923) on longevity, Jennings (1939) remarks:

> Every gene (with seemingly few exceptions) plays an essential role in the life and development of every cell of the body. It is not surprising, therefore, that changing a single gene may so alter the cellular processes as to change the length of life.

Longer duration of life in the female over that of the male is also explained on the basis of heredity rather than environment, for it might be regarded as a constitutive element of females. To the female is attributed a lower metabolic rate; consequently, in females there is a slower rate of the gradual condensation of biocolloids, and also a retardation of the processes which render metabolism impossible. As a result of this retardation there is a slower production of a substance leading to old age and natural death.

In addition to the physiological processes which lengthen or shorten the life span of insects, it must be recognized that we have at work natural selection which regulates these physiological processes in some way, thus prolonging the life of the female to insure oviposition which is an advantage to the race and curtailing useless existence of males which become sterile after a few weeks.

It must be borne in mind that the results obtained under the conditions of these investigations may be altered or perhaps even reversed under other conditions, and it would be interesting to investigate whether the results were comparable to the above if the adults of *Habrobracon* were sub-

jected to a starvation diet. It is not improbable that cohabitation of males and females would decrease the mean duration of life of both sexes.

Summary

The results of the investigations of duration of life of 1,180 wild-type and 1,366 double mutant short-wing, white-eye individuals of the parasitic wasp, *Habrobracon juglandis* (Ashmead), under constant conditions of about 30° C. temperature, about 79.5 per cent relative humidity, with a population density of four males or four females, and a food diet of honey-water, were as follows:

(1) The mean duration of life of wild-type male was 24.093 ± 0.334 days, and that of the wild-type female was 29.250 ± 0.517 days. The mean duration of life of the female exceeded that of the male by 5.157 days. The difference was statistically significant.

(2) The mean duration of life of the double mutant male was 19.946 ± 0.259 days as compared with that of the double mutant female which was 23.991 ± 0.407 days. The mean duration of life of the female exceeded that of the male by 4.045 days. The difference was significant.

(3) The mean duration of life of the wild type male exceeded that of the double mutant male by 4.147 days. The difference was statistically significant.

(4) The mean duration of life of the wild -type female exceeded that of the double mutant female by 5.259 days. The difference was statistically significant.

(5) The mean duration of life of the wild-type, sexes combined, was 26.186 days, and the mean duration of life of the double mutant, sexes combined, was 21.566 days. The difference in their mean duration was 4.620 days, and this difference, too, was statistically significant.

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